Nanostructured systems for enzyme immobilization

PhD Thesis Book

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1. Introduction and aims

The dynamic growing industries and technologies require more effective, selective and sustainable synthetic processes. The commonly used chemical techniques are not always fulfilling the higher demands and new requirements. Since the biotechnology plays a crucial role in several industrial and development processes.

The further development of different industries became hardly impossible without having biotechnological processes e.g. for pharmaceutical, fine chemical, food-, textile industry or even for modern medical science.\(^1\)

Enzymes means crucial factors in biotechnology with them several enantiomer pure chemicals can be produced. These pure chemicals are essential for pharmaceutical, food and cosmetics industry.\(^2,3\) The enzymes are getting more and more important in modern medicinal methods and diagnostic equipments their usage is also inevitable in future therapeutic application. The enzymes are often used in soluble homogenous systems. The isolation of enzymes from solutions is very difficult, recycling and renewal of enzymes is impossible. Besides of these concerns it may be a serious issue that enzymes are not tolerating the conditions within biochemical reactions hence enzyme activity can be lost prior usage. The different enzyme immobilization techniques could be the solution for these biochemical challenges and economical concerns.

Nowadays there are numerous enzyme immobilization processes which can suit to the condition of different bio transformation. The immobilization can significantly enhance the stability of enzymatic products and their handling.\(^4\)

The two or three dimensional nano sized materials are getting more important in the field of modern biotechnology. The structural and functional nano materials can be potential carrier platform with stabilizing effects to several enzyme molecules without limiting their natural functionality. The enzymes immobilized by nano structured materials can provide promising solutions for industrial biotransformation and development of selective biosensors.\(^5\)

My PhD thesis focused on the development of efficient nanomaterials as nanoporous sol-gel matrices, cross-linked enzyme systems, nanofibers, carbon nanotubes and magnetic nanoparticles for enzyme immobilization. The aim of the experiments was to perform stable, easy-to-handle and reusable biocatalyst, which can be applied in batch or continuous flow mode biotransformation. The immobilization of lipase and ammonia-lyase were investigated, which are relevant biocatalysts in enantioselective synthesis for pharmaceutical or food industry and in biomedical application also. Our aim was to fine-tuning the properties of sol-gel entrapped lipases by various organosilane precursors and to modify the surface of carbon nanotubes for covalent binding of ammonia-lyase, to perform well applied biocatalysts in batch and continuous-flow reactors. Bisepoxide as a novel “missing” cross-linker for enzymes and electrospun nanofibers for entrapment of lipases were also investigated as efficient immobilization techniques. Surface functionalized magnetic nanoparticles were also synthetized for enzyme immobilization. Microfluidic Lab-on-a-Chip filled with enzyme covered magnetic nanoparticles was also developed to perform rapid and efficient biotransformations.

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2. Background

2.1. Enzyme immobilization techniques
The aim of enzyme immobilization is to improve the enzyme stability, handling and storage properties. Nowadays the enzymes are used for many applications where their native form is impossible. The enzyme reaction based biosensors used for certain therapies can be highlighted as good example but the enzymes applied in industrial biotransformation are also requiring the stable reusable bio catalysts. These demands can be achieved by properly immobilized enzymes.° There are several methods for effective immobilization of enzymes where the enzyme molecules can be connected with solid carrier, embedded in open matrix or they can be cross linked by physical or chemical interaction.

2.2. For enzyme immobilization active nano materials
Usage of nano materials id getting more and more important e.g. sensors in electric circuit, biosensors, automotive and pharmaceutical industry, contrast materials in different therapy.° Fine-tuned interactions can be created between enzymes and nano materials. The benefit of enzyme immobilization by nano materials is that very high effectiveness can be achieved due to high specific surface area of nano materials. Several nano particles have been used already successfully for enzyme immobilization creating different nano structured systems e.g. nano porous materials, nanofibers nano tubes, nano materials, nano hollow fiber materials.®

2.2.1. Sol-gel networks for enzyme immobilization
Sol-gel process is able to immobilize enzymes in a way that during the network creating process is embedding enzyme molecules. During immobilization the enzyme is added as first step to initial sol material. With this procedure the structure of enzyme is frozen defined its catalytic function. Enzymes embedded into sol-gel network, so called xerogels are mechanically stable, fine powder materials.° According to current researches the composition of polymer matrixes are essential for immobilized enzyme activity and selectivity for example. Experiments showed, that alcy-substitued organosilane precursor have beneficial effect on the sol-gel entrapped enzymes.°

2.2.2. Protein matrixes
Enzymes are connected by covalent binding during creation of protein based matrixes. The cross links between the enzymes are created by bi-functional agents. This method is leading to a cross linked protein based polymer which is insoluble. However the material does not contain solid carrier but it can be defined as nano structured immobilized enzyme. Glutaraldehyde is the most common used cross-linker, can forms Schiff-base with amino groups of the enzyme in rapid and easy way. The advantage of the method is simple, cost effective, generally applicable for most of the enzymes. Cross linking can be done directly from fermentation media. With this method immobilized enzyme can be created without cleaning or intermediate materials.°
The bio catalytic effect of cross linked enzymes depend on structural changes of enzyme coming from covalent binding. Another important factor is the size and geometry of nano channels (1-20 nm) between enzymes which defines the free flow property of biocatalysts. Disadvantage of this method is that enzymes located on outer surface can contact to substrate without any problem, while material transport of enzymes located deeper in the matrix can be difficult. Further problem could be that bindings of cross linked agents have negative effect on enzyme structure resulting enzyme inactivity.

2.2.3. Nano fibers

It is possible to create nano fibers by electro static fiber production. Enzymes can be immobilized inside or outside of these nano fibers. During immobilization inside of nano fibers the enzyme solution is mixed with the polymer solution after nano fibers are created from the mixture. In this case the enzymes are embedded in situ between the polymer chains and inside the fiber material. This method can be influenced by polymer material and its solution hence the enzymes are not tolerating any materials except their sensitively buffered media. The most effective materials are the biocompatible water soluble polymers (PVA) or gelatins, these materials can create homogenous systems with enzymes. Enzyme immobilization insides the fiber material provides higher security stability and better quality for enzyme functionality comparing immobilization on outer surface of fiber materials.¹¹

2.2.4. Nanotubes

The application field of carbon nano tube is very promising due to its mechanical, thermal and structural properties. Nowadays they can be found in electronics, optic fibers and in different sensors. The carbon nano tubes are widely used enzyme carriers especially for in development of bio fuel cells. Single wall and multi walled nano tubes are also widely used for enzyme immobilization. Advantage of single wall nano tube is the high specific surface area which makes them effective carrier materials for enzymes but the multi walled tubes are also used due to their easier dispergation property. The enzyme can be immobilized on surface of nano tubes by covalent and secondary binding. The advantage of enzyme adsorption is that it can preserves both original behavior of the enzymes and the support also. The adsorption can be performed by H-bridges, hydrophobic or π‒π interactions. However the adsorptive enzyme immobilization keeps the optimal conformation of enzyme but it could dissolve easily during application which empties catalyst quickly. The covalent binging provides more robust and long lasting connection between enzyme and nano tube.¹²

2.2.5. Magnetic nanoparticles

The nano particles are solid materials but due to their small size they narrow the properties of homogenous liquid phase reactions therefore by their usage different transport processes can be completed effectively. The nano particle can be difficulty isolated from reaction mixture since the filtration or centrifugation of stable colloid system is a real challenge. The isolation can be managed by the magnetic function of the nano particles.

The nano particles can be mobilized, directed and isolated by magnetic field. By their very advantageous property i.e. magnetic function of nano particles (MNP) they can be very promising materials. The magnetic nano particles can immobilize enzymes if the nano particles have adequate geometry and surface property. The immobilized enzyme narrows the dissolved protein behavior. Enzymes immobilized to magnetic nano particles can be very promising therefore a lot of research studies are being done in this section. On magnetic nano particles successfully immobilized enzymes can be used for diagnostic purposes e.g. blood sugar measurements, stereo selective bio transformations.\(^1\)

3. Experimental session

3.1. Sol-gel immobilization of lipase

TRIS-HCl buffer (0.05 M, pH 7.5, 390 µL), PEG solution (4% w/v, 200 µL) and IPA (200 µL) were added to a 20 mL glass vial and the mixture was shaken at 1000 rpm at room temperature for 10 minutes. Lipase AK (50 mg), the mixture of silane precursors (3 mmol, compositions see in Tables 1-3) and NaF solution (1 M, 100 µL) were added while continuous shaking was maintained. To complete the polymerization process the mixture was shaken for 12 hours at room temperature. The solid formed was washed with IPA (7 mL), distilled water (5 mL), IPA (5 mL) and n-hexane (5 mL). The resulting white powder was dried at room temperature for 24 h and stored at room temperature.

3.2. Preparation cross-linked phenylalanine ammonia-lyase biocatalysts

The phenylalanine ammonia-lyase enzyme solution (1 ml, 2 mg ml\(^{-1}\), in TRIS buffer 0.1M, pH 8.8) and ethanol (3 ml, anhydrous) as precipitant were shaken at room temperature for 10 min. GA solution or GDE (80 µl, 2%) were added to the mixture with continuous shaking (450 rpm). After 24 h the mixture was centrifuged (2200 g, 15 min, 8°C), the supernatant decanted and the residue washed three times with TRIS buffer (suspension in 10 ml, 0.1M, pH 8.8 followed by centrifugation: 2200 g, 15 min, 8°C) and once with ethanol (suspension in 10 ml followed by centrifugation: 2200 g, 15 min, 8°C). Prior to use CLEAs were kept under ethanol (2 ml) at 4°C.

3.3. Entrapment of lipase in electrospun nanofibers

Lipases (Candida antarctica lipase B, CaLB), enzyme solution (100 µL; 35.5 mg mL\(^{-1}\) of lipase) was prepared in sodium phosphate buffer (0.1 M, pH 7.5). The enzyme solution and the corresponding additive (5 µL) were added to PVA solution (675 mg; 10 m/m% PVA in distilled water). The PVA solution containing the lipase and the bioimprinting agent was sonicated for 25 min at 22 °C. For electrospinning the lipase-containing PVA solutions were transferred into a syringe (1 mL) and were fed to the emitter at 0.8–1.00 mL h\(^{-1}\) rate using a syringe pump (Aitecs SEP-10S Plus, Vilnius, Lithuania). The distance between the collector and the emitter (with 0.7 mm internal diameter) of the electrospinning equipment was 10 cm. Constant voltage ranging from 20–30 kV was applied to the emitter using a direct current power supplier (NT-35 High Voltage DC Supply MA2000, Nagykanizsa, Hungary). Electrospun fibers were collected on aluminum foil affixed to the collector. Electrospinning experiments were conducted at room temperature (22±2 °C).

3.4. Covalent binding of phenylalanine ammonia-lyase on to functionalized single-walled carbon nanotubes

1 ml PAL solution (2.0 mg in 1 mL of 0.1 M Tris buffer, pH 8.8) was added to 20 mg functionalized single-walled carbon nanotubes (SwCNT, carboxy-functionalized: SwCNT-A, epoxy-functionalized: SwCNT-B) and the mixture was shaken at room temperature at 1350 rpm, overnight. After the PAL-immobilization, the resulted biocatalyst (SwCNTCOOH-PALII) was filtered off on a membrane filter and washed with distilled water (3×10 mL).

3.5. Immobilization of phenylalanine ammonia-lyase on to magnetic nanoparticles

108 mg epoxy/phenyl-functionalized –magnetic nanoparticles (MNP) was added to TRIS buffer (3 mL, 0.1 M, pH 8.8) and dispersed by ultrasonication (35 kHz, 20 min). The MNP suspension was added to the PAL solution (3 mg mL\(^{-1}\), in TRIS buffer: 4 mL, 0.1 M, pH 8.8) and the mixture was shaken for 24 h (25 °C, 450 rpm). The MNP-PAL particles were fixed at the bottom of the flask with a neodymium magnet and the supernatant was decanted. The MNP-PAL preparation was washed – by a re-suspension - magnetic fixing - decantation sequence in each washing steps – with TRIS buffer (3 × 4 mL, 0.1 M, pH 8.8) and with ethanol (4 mL). The MNP-PAL was dried in vacuum at room temperature for 2 h.

4. Results

4.1. Entrapment of lipases in nanoporous sol-gel matrices

The disubstituted dimethyldiethoxysilane (DMDEOS), methyl(phenyl)diethoxysilane (MPDEOS) and diphenyldiethoxysilane (DPDEOS) were used in binary silane precursor systems in combination with tetraethoxysilane (TEOS) for the immobilization of lipase from *Pseudomonas fluorescens*. The lipase biocatalysts were tested in the kinetic resolution of racemic-1-phenylethanol (rac-1) in organic media (Figure 1.).

![Figure 1. Kinetic resolutions for testing of lipase biocatalysts](image)

The use of disubstituted dialkoxy silanes with different substituents combined with TEOS as silane precursors may significantly influence the porosity and the hydrophobicity of the sol-gel matrices obtained which may play a significant role in the final biocatalytic properties of the entrapped lipases. As expected, the activity of lipase improved in all of these sol-gel matrices (\(Y_A > 100\%\)), while the high degree of selectivity was retained. Taking into account also the specific activity, the DMDEOS containing sol-gel matrix was much more efficient (\(YA = 994 \%\)) for DMDEOS/TEOS system DPDEOS/TEOS and MPDEOS/TEOS systems, respectively. The enantiomeric excess was also high in the case of DMDEOS (\(ee_{(R)-2a} = 99,3 \%\))
Table 1. Biocatalytic properties of Lipase AK entrapped in binary sol-gel systems (1:1 molar ratio of the silane precursor/TEOS; with rac-1a, reaction time 4 h in n-hexane:THF 2:1)

<table>
<thead>
<tr>
<th>Silane precursors</th>
<th>c (%)</th>
<th>ee(R)-2 (%)</th>
<th>E</th>
<th>U_B (U g⁻¹)</th>
<th>Y_A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native lipase</td>
<td>29.7</td>
<td>97.9</td>
<td>&gt;100</td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td>TEOS</td>
<td>27.5</td>
<td>98.3</td>
<td>&gt;200</td>
<td>9.1</td>
<td>350</td>
</tr>
<tr>
<td>TEOS/PTEOS</td>
<td>30.6</td>
<td>98.3</td>
<td>&gt;100</td>
<td>10.5</td>
<td>360</td>
</tr>
<tr>
<td>TEOS/OTEOS</td>
<td>49.4</td>
<td>98.3</td>
<td>&gt;200</td>
<td>17.0</td>
<td>374</td>
</tr>
<tr>
<td>TEOS/DPDEOS</td>
<td>14.4</td>
<td>98.8</td>
<td>&gt;100</td>
<td>5.0</td>
<td>750</td>
</tr>
<tr>
<td>TEOS/MPDEOS</td>
<td>28.9</td>
<td>98.8</td>
<td>&gt;200</td>
<td>8.2</td>
<td>900</td>
</tr>
<tr>
<td>TEOS/DMDEOS</td>
<td>47.2</td>
<td>99.3</td>
<td>&gt;200</td>
<td>16.3</td>
<td>994</td>
</tr>
</tbody>
</table>

For practical applications of immobilized enzymes, reusability is of foremost importance. For testing the recyclability of our best DMDEOS containing sol-gel biocatalysts were compared to native lipase and a conventional binary sol-gel lipase (iBTMOS/TMOS 5:1) in repeated kinetic resolutions of rac-1 (see in Fig. 1). Each biocatalysts was reused 7 times for 4 hours. We found that native Lipase AK not only suffered from the highest mass lost (~25% average mass lost in each cycle), the specific activity of native lipase decreased also continuously and the enantiomer selectivity was also significantly reduced. All of our sol-gel preparations exhibited much better durability in repeated reaction cycles. Not only were the average mass losses of the sol-gel-AK biocatalysts significantly lower, but they retained much more of their original specific activity.

4.2. Cross-linked enzyme aggregates

Cross-linked PAL enzyme were prepared using GDE and GA differ in several aspects. For example, the cross-link with GDE (11 heavy atom-long chain with hydrogen bond donor and acceptor groups) is longer and more hydrophilic than that produced with GA under dilute, slightly basic conditions (5 carbon-long aliphatic chain). Moreover, the C–X bonds (X= NH, S, O) forming in GDE cross-linking, unlike the C=N bonds forming in GA cross-linking are not susceptible to hydrolysis. The mechanical stability of GDE and GA cross-linked PAL were investigated by analysis of their particle size distribution after strong ultrasonication (Figure 2.) GDE-based CLEAs exhibited a more uniform particle size distribution and better resistance to mechanical stress by ultrasonication than the corresponding GA-based biocatalyst. These differences may be explained by the different nature of cross-links with GA and GDE. GDE is more uniform than that of the cross-links generated with GA. Because the epoxide functions of GDE may form cross-links not only via the surface exposed amine functions of Lys residues but also via S- and O-atoms of Cys, Tyr, Asp, Glu, cross-link density may be higher in the GDE-based systems than in GA-based ones, especially in case of proteins having only few surface exposed lysine residues.

Figure 2. The effect of ultrasonication on the particle size distribution of cross-linked PAL biocatalyst by Glycerol diglycidyl ether(GDE) A) and glutaraldehide B)
The biocatalytic activity of GDE- and GA-cross-linked PALs were compared in the elimination reaction from DL-3 and addition reaction onto (E)-4 (Figure 3).

![Chemical structures](image)

**Figure 3.** PAL catalyzed ammonia elimination of DL-3 and ammonia addition of (E)-4

Recycling studies revealed remarkable stability differences between GA- and GDE-based PAL biocatalyst (Fig. 3). Whereas the GDE-based system retained significant portion of their initial activities after the third reaction cycles even in the alkaline medium of ammonia addition onto (E)-4 (Fig. 3; 6 M ammonia solution, pH 10.0), the GA-based system were found to be completely inactivated after the first cycle. Our results indicated that GDE cross-linking enhances not only the mechanical stability of the cross-linked PAL formed but also the operational stability compared to that of GA-based PAL.

![Conversion graphs](image)

**Figure 4.** The conversion of PAL catalyzed ammonia elimination from DL-3 A) and ammonia addition to (E)-4 B)

### 4.3. Entrapment of lipase in electrospun nanofibers

Lipase B from *Candida antarctica* (CaLB) entrapped in poly(vinyl alcohol) (PVA) nanofibers. Three types of additives, such as polyethylene glycols, non-ionic detergents and organosilanes, were investigated as bioimprinting molecules for the lipases. Polyethylene glycols and non-ionic detergents were already applied as substrate analogs exhibiting bioimprinting effects in sol-gel systems, but their effect in PVA entrapment has not been investigated yet. In a previous study, the bioimprinting effect of several organosilanes applied as silane precursors to form the sol-gel matrix was considered assuming their substrate analog function. In this study the bioimprinting effect of organosilanes such as phenyltriethoxysilane (PTEOS), octyltriethoxysilane (OTEOS) has been clearly demonstrated. The bioimprinted nanofibrous lipase biocatalysts were tested in the kinetic resolution of 1 phenylethanol *rac*-1 (Figure 1.).
Table 2. Effect of additives on the biocatalytic properties of *Candida antarctica* lipase B (CaLB) entrapped in PVA nanofibers in the kinetic resolution of rac-1, reaction time 2 h

<table>
<thead>
<tr>
<th>Additives</th>
<th>c (%)</th>
<th>ee&lt;sub&gt;(R)-2&lt;/sub&gt; (%)</th>
<th>E (-)</th>
<th>U&lt;sub&gt;B&lt;/sub&gt; (U g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Y&lt;sub&gt;A&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native CaLB</td>
<td>8.3</td>
<td>98.7</td>
<td>&gt;100</td>
<td>5.7</td>
<td>100</td>
</tr>
<tr>
<td>-</td>
<td>15.0</td>
<td>99.8</td>
<td>»200</td>
<td>10.4</td>
<td>3605</td>
</tr>
<tr>
<td>Brij 30</td>
<td>13.0</td>
<td>99.8</td>
<td>»200</td>
<td>9.0</td>
<td>3124</td>
</tr>
<tr>
<td>Tween 80</td>
<td>16.6</td>
<td>99.9</td>
<td>»200</td>
<td>11.4</td>
<td>3983</td>
</tr>
<tr>
<td>PEG 400</td>
<td>21.3</td>
<td>99.9</td>
<td>»200</td>
<td>14.7</td>
<td>5118</td>
</tr>
<tr>
<td>PEG 1000</td>
<td>14.8</td>
<td>99.5</td>
<td>»200</td>
<td>10.2</td>
<td>3553</td>
</tr>
<tr>
<td>TEOS</td>
<td>8.8</td>
<td>98.6</td>
<td>&gt;100</td>
<td>6.1</td>
<td>2118</td>
</tr>
<tr>
<td>PTEOS</td>
<td>8.6</td>
<td>99.5</td>
<td>»200</td>
<td>12.8</td>
<td>4460</td>
</tr>
<tr>
<td>OTEOS</td>
<td>18.8</td>
<td>99.5</td>
<td>»200</td>
<td>12.9</td>
<td>4499</td>
</tr>
<tr>
<td>DMDEOS</td>
<td>12.3</td>
<td>99.7</td>
<td>»200</td>
<td>8.5</td>
<td>2957</td>
</tr>
</tbody>
</table>

Even the simple PVA nanofiber-entrapped CaLB biocatalysts showed thirty times higher activity than the non-immobilized CaLB powder (Table 2.). Addition of the bioimprinting agents further enhanced the catalytic properties of PVA-entrapped CaLB in each case. Like with the other two lipases, PEG 400 proved to be an especially efficient additive with CaLB resulting in a biocatalyst with more than fifty times higher specific activity as the native powder with enhanced ee<sub>(R)-2</sub> of the product (99.9 %; compared to 98.7 % with the native powder). To understand the effect of substituted organosilanes, OTEOS, PTEOS and DMEOS were docked into the active site of CaLB and the spatial location of organosilanes were compared to the arrangement of Tween 80 (T80) substrate analogue present in the active site. In case of OTEOS it is clearly visible, that the Si atom of a partially hydrolyzed derivative can occupy a location next to the catalytic serine side-chain and the octyl chain – similarly as Tween 80 – can keep the small lid-like loop in open position (Figure 3 A). PTEOS can also fit to the active site and the planar aromatic ring occupying a similar position is able to maintain the opened, active conformation (Figure 3 B). Although DMDEOS could be docked also with its Si atom close to the catalytic triad, it is not large enough to influence the movement of the lid-like loop, thus cannot force an open conformation efficiently.

**4.4. Covalent binding of phenylalanine ammonia-lyase onto functionalized carbon nanotubes**

PAL enzyme was immobilized onto two different surface functionalizes single-walled carbon nanotubes (SwCNT). The carboxy-functionalized nanotubes SwCNT-A are well known for enzyme immobilization, thus it was tested in the covalent binding of PAL. While glycerol diglycidyl ether (GDE) was an efficient cross-linker for PAL, it was applied for the functionalization of SwCNT also to perform epoxy rings of the surface (SwCNT-B). PAL immobilized in carboxy-functionalized (SwCNT-A-PAL) and on epoxy-functionalized (SwCNT-B-PAL) nanotubes, than tested as biocatalysts in the ammonia elimination from DL-3 and ammonia addition to (E)-4 (Figure 3.) in batch mode at room temperature. SwCNT-A-PAL showed high conversions (in the kinetic resolution from racemic DL-3 close to the theoretically possible 50 % conversion) and could be reused several times without significant loss of their initial activity (Table 3). Not surprisingly, under the much harsher conditions required for ammonia addition to 2 (6 M ammonia, at pH 10) the SwCNT-PALs were much less durable. Among the two forms, SwCNT-B-PAL enabled the highest number of recycling and retained more than 85 % of the initial activity even after 4 cycles (Table 3.)
Table 3. Conversion of the reactions catalyzed by the two forms of PAL immobilized on functionalized SwCNTs, reaction time 17 h

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Elimination</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DL-3, TRIS-puffer</td>
<td>(E)-4, 6 M NH3</td>
</tr>
<tr>
<td></td>
<td>c (%)</td>
<td>c (%)</td>
</tr>
<tr>
<td>SwCNT-A-PAL</td>
<td>SwCNT-B-PAL</td>
<td>SwCNT-A-PAL</td>
</tr>
<tr>
<td>1</td>
<td>48.4</td>
<td>49.2</td>
</tr>
<tr>
<td>2</td>
<td>49.2</td>
<td>48.5</td>
</tr>
<tr>
<td>3</td>
<td>49.0</td>
<td>48.8</td>
</tr>
<tr>
<td>4</td>
<td>49.3</td>
<td>47.4</td>
</tr>
<tr>
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<tr>
<td>6</td>
<td>43.7</td>
<td>42.4</td>
</tr>
<tr>
<td>7</td>
<td>42.5</td>
<td>42.3</td>
</tr>
</tbody>
</table>

Continuous-flow ammonia addition to (E)-4 was studied in a microreactor system including a biocompatible packed-bed PTFE column filled with SwCNT-B-PAL. The bioreactor unit was placed in the column thermostat of a HPLC system with full control of pressure and temperature (Figure 5).

![Figure 5](image)

Figure 5. PAL catalyzed ammonia addition on to (E)-4 in continuous-flow microreactor

As expected, the specific reaction rate with SwCNT-B-PAL in the continuous-flow reactor was significantly higher than that in the batch reaction. Next, the influence of the temperature on the ammonia addition reaction to 2 was investigated in the temperature range of 30–80 °C (Figure 6). Notably, also in a continuous-flow reactor conversions remained constant even after 72 h of operation and at temperatures up to 60 °C, indicating the remarkably improved durability of SwCNT-B-PAL in 2 M ammonia. Since the conversions depend primarily on the activity of the biocatalyst, a maximum value of the conversions at a so called “optimum temperature” was expected. Accordingly, activity of immobilized PAL increased when the temperature of the continuous-flow system was raised from 30 to 40 °C. Unexpectedly, by increasing the temperature beyond 40 °C, a local minimum of the initial conversion was observed at around 50 °C. This is why at 50 °C the conversion was significantly lower than at 40 °C or even at 30 °C. This apparent decrease of PAL activity at 50 °C cannot be attributed to irreversible thermal inactivation, because the level of activity at 50 °C remained stable over 72 h. This was also indicated by the run at 60 °C with conversion remaining stable over 72 h and higher than those observed in runs between 30 and 50 °C.

![Figure 6](image)

Figure 6. Temperature effect between 30 – 80 °C on the long term stability (B) in ammonia addition to (E)-4 catalyzed by SwCNT-B-PAL in a continuous-flow microreactor.
4.5. Investigation of phenylalanine ammonia-lyase in ammonia elimination of a new acyclic amino acid by continuous flow MagneChip device

Phenylalanine ammonia-lyase (PAL), in many organisms, catalyzes the deamination of L-phenylalanine (Phe) to (E)-cinnamate by the aid of its MIO prosthetic group. By using PAL, immobilized on magnetic nanoparticles and fixed in a microfluidic reactor with an in-line UV detector, we first demonstrated that PAL can catalyze the ammonia elimination from the acyclic propargylglycine (PG) to yield (E)-pent-2-ene-4-ynoate indicating new opportunities to extend the MIO-enzyme toolbox towards acyclic substrates. Deamination of PG, being acyclic, cannot involve a Friedel-Crafts-type attack at an aromatic ring. The reversibility of the PAL-reaction, also demonstrated by the ammonia addition to (E)-pent-2-ene-4-ynoate yielding enantiopure L-PG, contradicts the proposed highly exothermic single-step mechanism. Computations on the QM/MM models of the N-MIO intermediates from L-PG and L-Phe in PAL, showing similar arrangements within the active site, support a mechanism via the N-MIO intermediate. In microfluidic systems the protein-coated MNPs can either flow together with the liquid, or can be anchored by a magnet at definite site(s) while the free flowing fluid passes the region(s) where the particles are fixed.

![Figure 7. Ammonia elimination from DL-propargylglycine in a light protected microfluidic reactor with multiple magnetic-cells filled with PAL immobilized on MNPs and equipped with in-line UV-Vis detector (reaction in D$_2$O at pD 8.8, 37 °C). The progress of the reaction was followed by full UV-spectra.](image)

Magne-Chip was used for the microscale biotransformation of DL-propargylglycine in sodium carbonate-buffered D$_2$O at pD 8.8 (Figure 7.). For quantification of the product formation in the Magne-Chip device by in-line UV detection of the product. This magnetic LoC-device with in-line UV-detection enabled to detect the formation of (E)-pent-2-ene-4-ynoate at 242 nm and produced measurable quantities of the product for recording $^1$H-NMR spectra without any work-up. Besides the significant increase of the UV-signal at 242 nm (up to A= 1.2) in the on-line UV-cell (Figure 1), the appearance of olefin hydrogen signals in the $^1$H-NMR spectrum of the reaction mixture [at δ= 6.34 ($d$) and 6.85 ($d$) ppm] indicated unambiguously the formation of (E)-pent-2-ene-4-ynoate.
5. Novel scientific findings - Thesis

1. We described for the first time, that disubstituted organosilanes (dimethyldiethoxysilane diphenyldiethoxysilane and methyl-phenyldiethoxysilane) are suitable precursors to form nanoporous sol-gel matrices for immobilization of enzymes by entrapment. The sol-gel Pseudomonas fluorescens lipase entrapped within a matrix from disubstituted organosilanes showed enhanced biocatalytic activity in the kinetic resolution of racemic secondary alcohols. We proved, that the sol-gel entrapped lipases can preserve their biocatalytic activity and enantioselectivity in seven reaction cycles in batch mode, and the sol-gel biocatalysts are applicable in continuous flow-systems as well. [I]

2. We proved for the first time, that a bisepoxide can be used successfully as cross-linker for enzyme immobilization. Pseudomonas fluorescens, Pseudomonas cepacia, Candida antarctica B lipases and Petrosenilium crispum phenylalanine ammonia-lyase cross-linked by bisepoxide provide a novel, nanostructured protein matrices, which have stable and homogenous morphology. Examples of three lipases and an ammonia-lyase demonstrated that enzymes cross-linked by bisepoxides were suitable biocatalysts for selective biotransformations in aqueous as well as in organic media. [II]

3. We immobilized successfully three enzymes, Pseudomonas fluorescens, Pseudomonas cepacia, Candida antarctica B lipases, by entrapment of in poly(vinyl alcohol) (PVA) nanofibers using and optimizing electrospinning technique. We demonstrated for the first time, that additives acting as substrate analogue could enhance the biocatalytic properties of lipases entrapped in PVA fibers. We proved, that several organosilanes (phenyltriethoxysilane and octyltriethoxysilane) and their derivatives could act substrate analogues and fix the active conformation of lipases during entrapment in PVA nanofibers.[III]

4. For the first time, we performed the covalent immobilization of phenylalanine ammonia-lyase on surface-functionalized carbon nanotubes. We proved, that immobilization of PAL by the novel epoxy-functionalized nanotubes obtained by glycerol diglycidyl resulted in more stable biocatalyst, than direct attachment to the simple, well-known carboxyl functionalized carbon nanotubes. The ammonia addition onto 2-(thiophen-2-yl)acrylic acid demonstrated, that PAL immobilized on epoxy functionalized nanotubes was suitable biocatalyst for synthesis of enantiopure unnatural amino acids in batch as well as in continuous-flow systems. [IV]

5. For first time, phenylalanine ammonia-lyase (PAL) was attached covalently to the surface tailored-size functionalized magnetic nanoparticles (MNP-PAL), which were ideally applicable in microfluidic Lab-on-a-Chip devices (MagneChip). We proved, that biocatalytic activity of MNP-PAL increased more than threefold in MagneChip, compared to the biotransformation in shake flask. [V, VI]

6. We demonstrated for the first time, that an acyclic non-aromatic amino acid, DL-propargylglycine (PG) was a substrate of phenylalanine ammonia-lyase (PAL). The ammonia elimination from this substrate performed in a microfluidic Lab-on-a-Chip (MagneChip) device
resulting unambiguously in the elimination product contradicted the hypothetical Friedel-Crafts type PAL mechanism, thus supporting the mechanism via the N-MIO intermediate. [VI]

7. Potential applications and future aspects

The results of this study represent novel nanostructured materials, which can be well applied for enzyme immobilization. Entrapment of lipase in nanoporous sol-gel matrices built from novel dissubstituted organosilanes and ammonia-lyase covalently attached on functionalized carbon nanotubes can provide a robust biocatalyst for enantioselective bitransformation in batch and continuous-flow reactors. Novel stable and uniform cross-linked enzyme catalyst can be performed by bisepoxides, which are more active, than the traditionally applied cross-linker as glutaraldehyde. Substrate analogue additives enhanced the biocatalytic properties of lipases entrapped in nanofibers via bioimprinting effect, can provide efficient biocatalyst for continuous flow membrane reactor. Surface functionalized magnetic nanoparticles are efficient nanocarriers for enzymes, which are suitable for microfluidic Lab-on-a-Chip devices (MagneChip). MagneChip filled with enzyme covered magnetic nanoparticles can be promising device to investigate biotransformations or to develop high-through-put biosensors for biomedical applications.

8. Publications

8.1. Publications on witch the thesis based


IV. F Ender, D Weiser, B Nagy, CL Bencze, C Paizs, P Pálovics, L Poppe, Microfluidic multiple cell chip reactor filled with enzyme-coated magnetic nanoparticles – An efficient and flexible novel tool for enzyme catalyzed biotransformations, J Flow Chem, 2015, accepted with minor revision, IF (2014): 1,878; I: - (part of WD: 25 %)

8.2. Other publications and presentations


Oral presentations:


Poster presentations: